Look at the size of those pores! Variation in stomatal behavior in *Brassica rapa* suggests link to circadian clock

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ABSTRACT
The circadian clock is an endogenous *c.* 24 hour timekeeping mechanism which regulates a number of plant processes. Stomata are leaf pores which open and close for water and gas exchange. In this study, I sought to examine whether stomatal behavior is influenced by the clock. To do so, I measured the stomatal aperture of 8 lines of *Brassica rapa* (*B. rapa*) with different circadian periods (the time it takes the clock to complete one full oscillation) 1 hour before dawn and 1 hour after dawn over three days. I hypothesized that plants with different periods would differ in mean stomatal aperture at each time point and the amount that aperture changed between the two timepoints. I found that the lines differed in their aperture and change in aperture, though those with similar period length did not necessarily demonstrate similar trends in aperture.

INTRODUCTION

Stomata are small pores in the leaf epidermis formed by two adjacent guard cells that open and close as guard cell turgor pressure decreases and increases, respectively (Zhang et al., 2018; Bergmann and Sack, 2007). Open stomata allow carbon dioxide to enter the leaf for photosynthesis, but at the expense of water escaping via transpiration. Typically, stomata are open during the day to take in carbon dioxide for photosynthesis, and closed at night to limit water loss. Stress may alter these patterns. For example, under drought conditions, abscisic acid (ABA) is produced. This reduces guard cell turgor pressure, closing stomata for improved water conservation (Kim et al., 2010; Lawson and Matthews, 2020; Bertolino et al., 2019). Identifying the key players of stomatal aperture regulation will advance breeding efforts to improve water use efficiency.

Mounting evidence suggests the circadian clock, an endogenous *c.* 24 hour (h) timekeeping mechanism, regulates stomatal function and behavior. (Chaix et al., 2016; Araujo et al, 2011; Hassidim et al., 2017; Simon et al., 2020). Circadian regulation also acts on many key developmental traits in plants (Greenham and McClung, 2015). As such, clock outputs tend to be of interest when identifying targets for plant breeding. Previous work has shown that soybean cultivars bred for optimal growth at specific latitudes differ in their circadian period, the time it takes to complete one full cycle of the clock (Greenham et al., 2017). This suggests that the clock helps optimize plant function for different environments by dictating when to anticipate a new day and prompting physiological processes to occur at the appropriate time. If a plant's circadian period is out of phase with external conditions, these processes may occur at times which reduce their efficiency because the plant does not anticipate dawn when it actually occurs. As a result, the plant's fitness is reduced. Identifying ways in which the clock influences plant function allows for better optimization of crops and agricultural practices.

To better understand the connection between circadian period and stomatal behavior, I sought to characterize the stomatal aperture of a pilot set of lines from a mapping population of advanced intercross recombinant inbred lines (AI-RILs). This population was previously generated by crossing two *B. rapa* subspecies: R500 (ssp. *trilocularis*, yellow sarson oilseed) and L58 (ssp. *parachinensis*, caixin) followed by several rounds of intercrossing. The resulting offspring were then selfed over many generations, yielding lines that are homozygous and can be used to map quantitative trait loci (QTL). Despite little initial variation between the parental lines, the AI-RIL population exhibits a broad range of circadian period lengths (Table 1). This population therefore allows for the assessment of stomatal behavior across a range of periods. The methodology developed in this study will later be used to map QTL, with the future goal of identifying the genetic basis of circadian-mediated stomatal function.

I hypothesized that genotypes with different periods would also have different levels of mean stomatal aperture before dawn and after dawn. For example, the stomatal aperture of long period plants might differ from that of short period plants. After measuring the mean stomatal aperture index (SAI) of 8 different AI-RIL genotypes 1 hour before and 1 hour after dawn over three days, I found that different genotypes varied in their degree of aperture and the magnitude of the change in aperture from before dawn to after. However, plants with similar length periods (long vs. short) did not conclusively exhibit similar behavior.

MATERIALS AND METHODS

Plant materials and growth

From a population of 186 AI-RILs, I selected 3 lines with short periods (8628, 8466, 8467), 3 with long periods (8482, 8518, 8659), and the 2 parental lines (R500, L58) to be grown and sampled (see Table 1 for period lengths). Seeds were sown on unfertilized germinating soil (BM2) in 2x3.5-inch pots and grown under short day (8h/16h light/dark) with a PPFD of 220 umol m^{-2} s^{-1} (400-700 nm spectra), 22℃ constant temperature, and 50-60% relative humidity for approximately 1 month between April and May (Fig. 1). The plants were entrained such that dawn, the moment the lights turned on, occurred at 10:00 AM. The lights coming on act as a zeitgeber, a cue which sets the timing of circadian period. Zeitgeber time (ZT) indicates time relative to when dawn occurs, with ZT(0) corresponding to dawn, ZT(-1) to one hour before dawn, and ZT(+1) to one hour after dawn.

Table 1. Period Length of Selected Genotypes Periods of selected advanced intercross recombinant inbred lines identified from leaf movement assay.

Category	Genotype	Period Length (h)
Short	8628	22.7
Short	8466	22.9
Short	8467	23.04
Medium	R500	24.0
Medium	L58	24.5
Long	8482	26.4
Long	8518	26.5
Long	8659	27.3

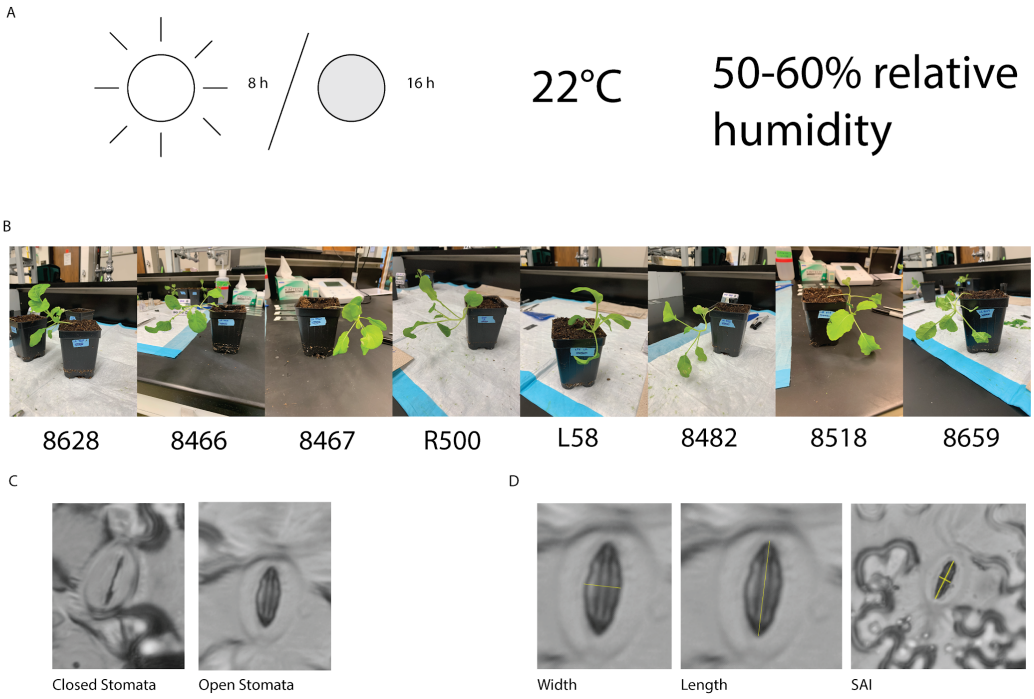

Figure 1. Overview of growth conditions, whole plant morphology, stomatal aperture measurements. (**A**) Plants were grown under short day (8h/16h light/dark) with a PPFD of 220 umol m^{-2} s^{-1} (400-700 nm spectra), 22℃ constant temperature, and 50-60% relative humidity for approximately 1 month between April and May. (**B**) Representative plants from each genotype. All were approximately the same developmental age at time of sampling. (**C**) Example of a closed (left) and open (right) stoma. (**D**) Sample measurement of width, length, and stomatal aperture index (SAI, width of stomata/length of stomata) from nail polish stomatal impressions.

Stomatal impressions

In order to measure mean stomatal aperture, 3 true leaves from one plant per genotype were sampled and nail polish was immediately applied at ZT(-1) or ZT(+1). At ZT(-1), plants were kept in the dark until they were sampled. Then, 3 true leaves from one plant were cut with a blade. For each day and timepoint of the experiment, a different plant was used. Each leaf was painted along the abaxial surface with a thin layer of clear nail polish (Sally Hansen Insta-Dri, shade 103 "Clearly Quick"). The nail polish was left to dry for 5 minutes. Then, the leaf was gently pressed with the abaxial side down onto a piece of double-sided tape (3M) adhered to a slide and left for 5 more minutes. The leaf was gently peeled away from the tape using forceps, leaving the nail polish impression adhered to the surface of the tape.

Stomatal image acquisition, quantification, and analysis

The abaxial stomatal impressions were viewed using an Olympus brightfield upright microscope equipped with an Amscope camera. AmLite imaging software (*ver.* 10.19.2020) on a MacOS (Sierra) was used to acquire the images. For best quality images, the microscope condenser was *c.* 80% closed. Randomized and non-overlapping images were acquired from the entire stomatal impression.

Fiji image analysis software (Version 2.1.0/1.53c) on a Mac OS (Sierra) was used to measure the width and length of each stomatal opening for 50 stomata per leaf (Fig. 1d). Dividing the width by the length yielded the stomatal aperture index (SAI), which was used as the main measure of aperture. Some leaves were excluded from analysis due to poor image or stomatal impression quality. To be included in analysis, 50 stomata per leaf had to be visible, with the edges of the stomatal opening sufficiently clear to identify where to begin and end measurements.

Three levels of comparison were made to assess the variation in stomatal aperture: between leaves from a single day and timepoint, between days for a single genotype, and between genotypes over all three days. For each leaf, the SAI from all the stomata measured was averaged to obtain a mean SAI (Fig. 2). For each day, the SAI from all stomata measured for a single genotype and timepoint was averaged. This yielded a mean SAI at ZT(-1) and at ZT(+1) on days 1, 2, and 3 for each genotype. The standard error of the mean was calculated for all of these, and paired t-tests were conducted to establish whether the mean SAI at ZT(-1) differed significantly from that at ZT(+1) on the same day. To compare between genotypes, stomata from all three days at one timepoint were pooled to obtain a mean SAI at ZT(-1) and at ZT(+1) for each genotype. The standard deviation was calculated for all of these, and paired t-tests were conducted to establish whether the mean SAI at ZT(-1) differed significantly from that at ZT(+1) for the same genotype.

All analyses were performed in RStudio (Version 1.1.456, R version 3.5.1) on a MacOS (Sierra). R scripts used are available upon request.

RESULTS

This pilot study sought to characterize the relationship between the circadian clock and stomatal function across select genotypes from a mapping population of plants. I assessed whether eight different lines of *Brassica rapa* (*B. rapa*) with a range of circadian periods differed in their stomatal aperture and change in aperture from 1h pre- and post-dawn. Differences between the lines might suggest a role for the clock in influencing stomatal function.

To characterize stomatal behavior in plants with varying circadian periods, I measured the stomatal aperture from 8 genotypes previously shown to exhibit short, medium, or long circadian periods via a leaf movement assay (Table 1). For each genotype, stomatal aperture was analyzed at two timepoints over three days: 1 h pre-dawn (ZT(-1)) and 1 h post-dawn (ZT(+1)) (Fig. 1).

Variation in stomatal aperture across leaves within a single plant

To examine differences in stomatal aperture, I compared leaves from a single plant, plants of the same genotype, and across genotypes. Within a single day, the SAI was fairly consistent from leaf to leaf for most genotypes. However, there was some variation (Fig. 2). In general, genotypes with a longer period seemed to vary more between leaves from the same plant on the same day than those with a shorter period. This was true at both ZT(-1) and ZT(+1).

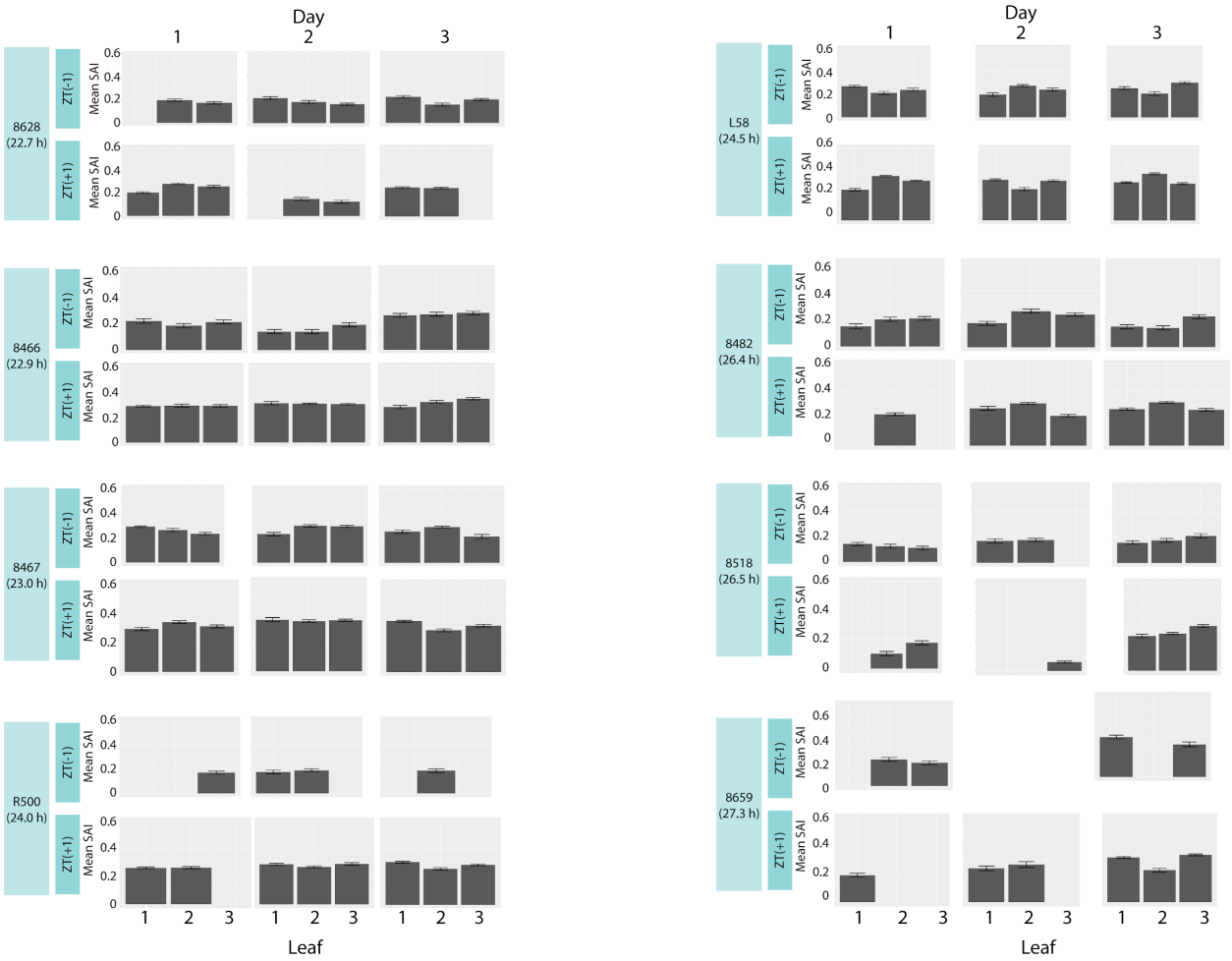

Figure 2. Stomatal Aperture by Leaf. Each bar represents the mean stomatal aperture index of stomata measured from a single leaf. Genotype of the leaf and the timepoint at which it was sampled are indicated in blue boxes. Day of sampling is indicated by 1, 2, or 3 along the top of the plot. Leaf number is indicated by 1, 2, or 3 along the bottom of the plot. SAI was calculated as the width of the stomata divided by its length. A higher SAI value indicates a more open degree of aperture, and a lower value SAI indicates a more closed degree of aperture. Missing bars indicate leaves excluded from analysis due to poor quality of stomatal impressions or images.

Magnitude of stomatal aperture and change vary between genotypes

For each day, stomatal aperture measurements were pooled from three technical replicates (i.e. three leaves were analyzed from each genotype per day) (Fig. 3). In six of the eight genotypes, the mean stomatal aperture index (SAI) increased from pre-dawn (ZT(-1)) to post-dawn (ZT(+1)) every day, as expected (Fig. 3). Genotypes 8628 and 8518 on day 2 were exceptions to this trend, exhibiting a decrease in aperture post-dawn (Fig. 3A, 3G). These inconsistencies may be partially explained by the exclusion of some leaves from analysis due to poor quality of either the stomatal impressions or the images acquired. As a result, some genotypes have fewer stomata measured (Fig. 3, Fig. 4A) or may be missing data on a certain day (Fig. 2, Fig. 3).

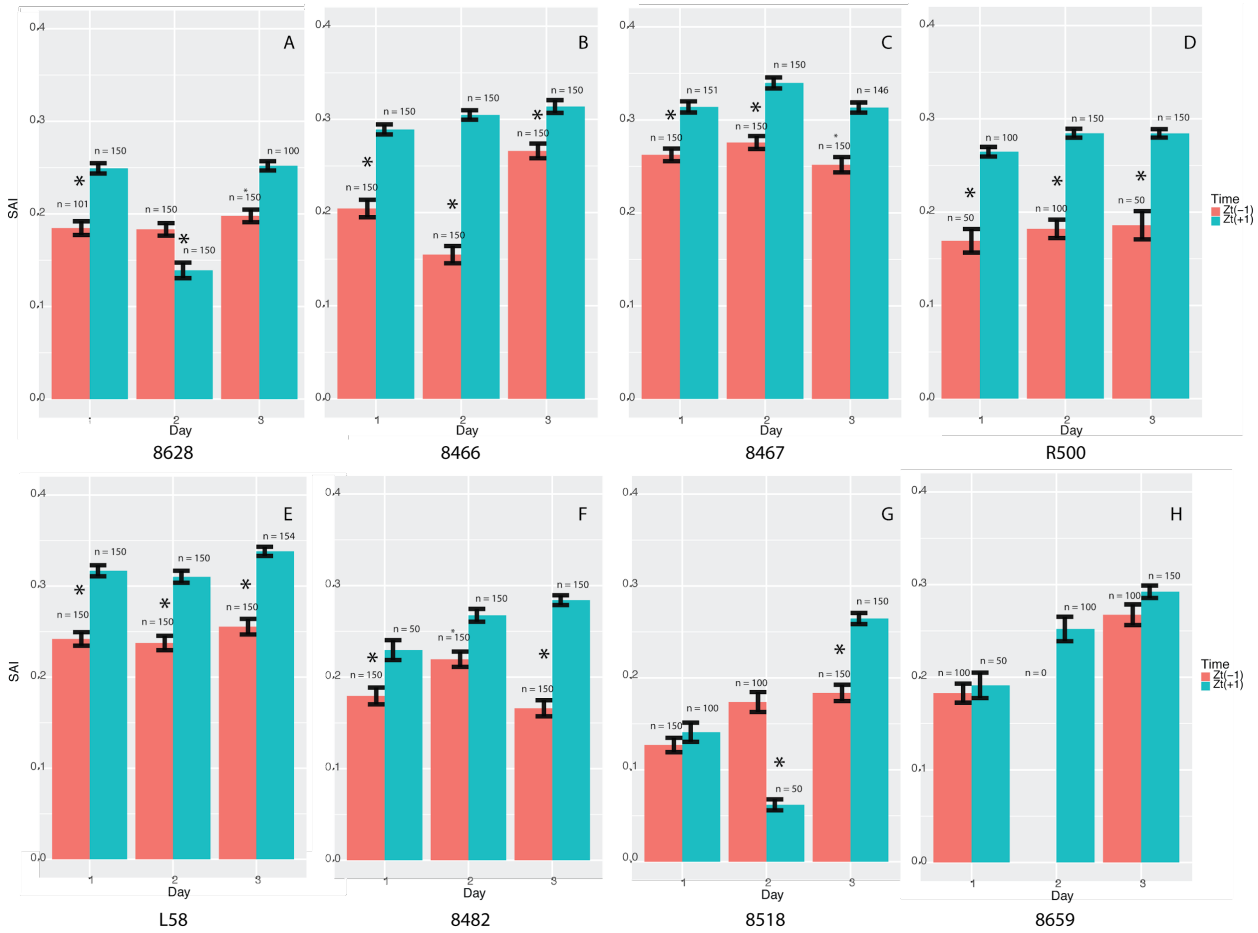

Figure 3. Change in Stomatal Aperture by Day. Each panel displays mean stomatal aperture index (SAI, width of stomata/length of stomata) of the genotype indicated at the bottom of the panel on day 1, 2, or 3 of the experiment as noted by the x-axis. Red bars correspond to plants sampled at ZT(-1), blue bars correspond to plants sampled at ZT(+1). Mean SAI was determined by pooling the SAI of stomata measured from 3 leaves on the same plant. Error bars depict the standard error of the mean. An asterisk indicates that the mean SAI at ZT(-1) is significantly different from that at ZT(+1) on the same day ($p < 0.05$) (Table S1). Sample size is indicated above each bar. Missing bars indicate leaves excluded from analysis due to poor quality of stomatal impressions or images. Genotypes appear in order of ascending period length.

I also observed that for some genotypes the mean SAI at ZT(-1) and ZT(+1) was fairly consistent across all three days, though there were some exceptions. Genotypes 8467, R500, and L58 maintained relatively constant trends in terms of both mean SAI and change in aperture across all 3 days. For other genotypes, change in aperture seemed to be somewhat more variable between days. However, due to the exclusion of some leaves, it was difficult to assess overall trends in mean SAI and aperture change post-dawn between genotypes from day to day. Therefore, I pooled all stomata from all three days (Fig. 4). This more clearly demonstrated that though stomatal aperture varied between genotypes, there was an overall increase in mean SAI from ZT(-1) to ZT(+1). Standard deviation was somewhat large, but consistent between genotypes and timepoints (Fig. 4A).

Genotype 8628, which has a period of 22.7 h, and 8518, with a period of 26.5 h, demonstrated the smallest changes in aperture from pre-dawn to post-dawn. Genotypes 8466 (22.9 h), 8467 (23.04 h), R500 (24 h), L58 (24.5 h), and 8482 (26.4 h) all exhibited similar magnitudes of change in aperture. The change was fairly large in all of these cases, although their respective means varied (Fig. 4A). Every genotype demonstrated a statistically significant change in aperture, with a significance threshold of $p < 0.05$ (Table S2).

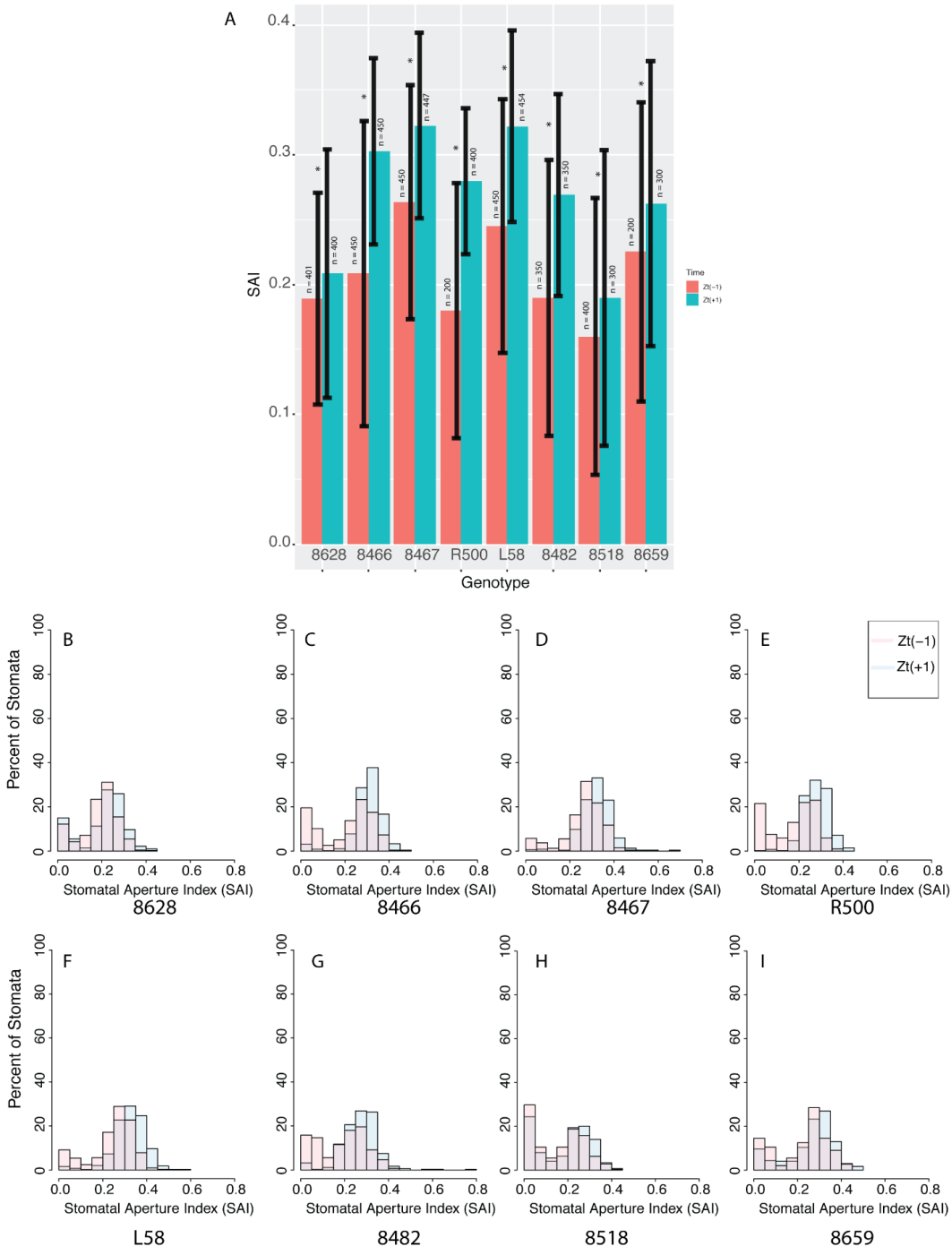

Figure 4. Change in Stomatal Aperture by Genotype. (A) Results of pooling measurements of all the stomata at the specified ZT for each genotype across all three days and calculating the mean SAI. Bars in red indicate mean

SAI at ZT(-1), and blue represents ZT(+1). Error bars depict the standard deviation. An asterisk indicates that the mean SAI at ZT(-1) is significantly different from that at ZT(+1) for the same genotype ($p < 0.05$) (Table S2). Sample size is indicated above each bar. Genotypes appear in order of ascending period length. **(B-I)** Distribution of the SAI of stomata pooled from all three days for each genotype at ZT(-1) (shown in red) and ZT(+1) (shown in blue). Each bar represents the percent of stomata (number of stomata/total number of stomata at specified ZT) with a certain SAI (indicated along x-axis). Genotypes appear in order of ascending period length.

To better visualize the distribution of stomatal aperture between ZT(-1) and ZT(+1), the pooled data points were plotted as overlapping histograms (Fig. 4B-I). Every genotype displayed an overall trend of increasing aperture from ZT(-1) to ZT(+1). The majority of the stomata at ZT(+1) fall further right along the x-axis than those at ZT(-1), which indicates a higher SAI value and greater aperture.

Interestingly, many genotypes exhibited non-Gaussian distributions of SAI at one or both timepoints. Particularly at ZT(-1), each genotype had a main grouping of stomata which fell to the left of the peak of the ZT(+1) distribution, but was still relatively near it in aperture. However, they also had a secondary peak farther to the left and close to or even greater in height than the main peak. This indicates that at ZT(-1), almost all stomata are more closed than at ZT(+1). However, some are very tightly closed, while others may be starting to open. Therefore, at ZT(-1) stomata tend to fall either very far left in the distribution or to the left of, but near, the typical aperture at ZT(+1).

DISCUSSION

Results from this pilot study indicate that stomata do increase in aperture from ZT(-1) to ZT(+1), as expected. For every genotype, the mean SAI at ZT(-1) was less than that at ZT(+1) when stomata from all three days of the experiment were pooled (Fig. 4A). A paired t-test found that this difference was statistically significant for every genotype, as well ($p < 0.05$, Table S2). These findings support what the existing literature has previously posited: stomata open in response to light (Araujo et al., 2011). The distribution of SAI at ZT(-1) further suggests that stomata actually begin to open prior to light stimuli, as the clock anticipates dawn and begins to induce a stomatal response (Fig. 4B-I).

It was also evident that stomatal aperture varies between genotypes. The lines differed in mean SAI at each timepoint as well as in the magnitude of change in mean SAI from ZT(-1) to ZT(+1). Mean SAI at ZT(-1) appeared more variable than that at ZT(+1), with a somewhat broader range of values amongst the genotypes. Post-dawn, some lines with different pre-dawn aperture tended to become more similar in terms of mean SAI. For example, the difference in aperture between 8466 and 8467 was greater at ZT(-1) than at ZT(+1). This was also true when comparing 8466 to R500, 8466 to L58, and 8467 to R500. However, aperture change actually appeared more variable from genotype to genotype than the means alone. It seems that the more salient difference in stomatal function between lines may be how robustly they respond to dawn, as demonstrated by the magnitude of their change in mean aperture. Overall, these data support the hypothesis that stomatal behavior varies between lines of *B. rapa* with different circadian periods.

It is less clear whether plants with long periods differ from those with short periods, or if plants with similar length periods behave alike. In general, category of period length (short,

medium, or long) did not seem to be a very strong predictor of mean SAI or aperture change. Two of the long period lines (8466 and 8467) showed similar changes to one another, and two of the short lines (8518 and 8659) were also alike in the magnitude of aperture change. However, the short genotype 8628 and the long genotype 8482 showed much less and much greater aperture change, respectively, than the other lines with similar period lengths. Genotype 8628 also demonstrated an unexpected decrease in aperture on day 2, though the sample size on this day was smaller than most of the others (Fig. 3). Looking at the distribution of stomatal aperture for the short period lines, 8628 further differs in that it demonstrated a secondary peak left of the mean at ZT(+1) that the other short lines only demonstrated at ZT(-1) (Fig. 4B). The anomalous decrease in aperture on day 2 likely contributes to this unexpected distribution. Overall, this day may account for the discrepancy between trends displayed by this genotype as compared to others with a short period.

Likewise, genotype 8482 contrasted from the other two long lines, demonstrating a greater increase in aperture. However, on days 1 and 2, this genotype exhibited a smaller increase in aperture (Fig. 3), more similar to the average of the other two long genotypes (Fig. 4A), with only day 3 showing a very large increase. Considering the effect this one day may have on the overall increase in aperture seen for this genotype (Fig. 4A), it seems conceivable that shorter genotypes generally exhibit greater changes in aperture in response to dawn than genotypes with longer periods. Also recall that when examining the distribution of the SAI of individual stomata (Fig. 4B-I), at ZT(+1) genotypes with longer periods tended to have a second, narrower peak further to the left of the bulk of the distribution, indicating that some stomata remained relatively closed even after dawn. This was not typical of lines with shorter periods. Overall, these data may suggest a less robust or delayed response to dawn in plants with longer periods under short day conditions.

However, the data do not support this hypothesis conclusively. The hypothesized relationship between long or short period and stomatal behavior is likely an oversimplification. It seems that there is some overlap between control of period length and of stomatal behavior, but that regulation of both of these traits is too complex for long or short period to accurately predict either mean stomatal aperture or the magnitude of aperture change post-dawn. For example, there may be some genes or gene products which influence both these traits and contribute to plants with different periods exhibiting differing stomatal behavior, but because there are others which regulate one trait or the other, period length alone cannot predict stomatal behavior. Future research could explore the genetic and molecular basis of these traits to better characterize their regulation and the extent to which they might be linked. QTL mapping in the full population of AI-RILs is one possible method by which to achieve this. By elucidating the connection between how these two traits are controlled, we may be able to identify targets for breeding to optimize stomatal behavior in conditions of drought, heat, or other stresses, while also avoiding disruption of the clock, which may lead to the unintended loss of other advantageous traits (Daszkowska-Golec and Szarejko, 2013; Greenham and McClung, 2015; Greenham et al., 2017).

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SUPPLEMENTARY MATERIALS

Table S1. Analysis of Significance of Aperture Change from ZT(-1) to ZT(+1) by Day
Results of paired t-tests between the mean SAI at ZT(-1) and ZT(+1) for each genotype on each day. A p-value of 0.05 was used as the threshold for significance. Genotypes appear in order of ascending period length.

Genotype	Day	p-Value
8628	1	4.344e-11
8628	2	5.386e-05
8628	3	8.424e-10
8466	1	1.626e-13
8466	2	< 2.2e-16
8466	3	7.58e-06
8467	1	2.656e-08
8467	2	1.527e-11
8467	3	1.733e-09
R500	1	1.593e-09
R500	2	< 2.2e-16
R500	3	5.67e-08
L58	1	1.164e-13
L58	2	1.382e-11
L58	3	5.358e-15
8482	1	0.0005788
8482	2	1.491e-05
8482	3	< 2.2e-16
8518	1	0.2913
8518	2	1.19e-15

8518	3	7.314e-13
8659	1	0.6275
8659	2	N/A
8659	3	0.05926

Table S2. Analysis of Significance of Aperture Change from ZT(-1) to ZT(+1) by Genotype
Results of paired t-tests between the mean SAI at ZT(-1) and ZT(+1) for each genotype after pooling stomata from all three days. A p-value of 0.05 was used as the threshold for significance. Genotypes appear in order of ascending period length.

Genotype	**p-Value**
8628	0.001983
8466	< 2.2e-16
8467	< 2.2e-16
R500	< 2.2e-16
L58	< 2.2e-16
8482	< 2.2e-16
8518	0.0005099
8659	0.0003978